

Manuscript EMBO-2013-87035

Convergent Regulation of Lysosomal Two-Pore Channel-2 by Mg²⁺, NAADP, PI(3,5)P₂ and Protein Kinases

Archana Jha, Malini Ahuja, sandip patel, Eugen Brailoiu and Schmuel Muallem

Corresponding author: Schmuel Muallem, NIDCR

Review timeline:

Submission date:	30 September 2013
Editorial Decision:	21 October 2013
Revision received:	22 November 2013
Editorial Decision:	02 December 2013
Revision received:	03 December 2013
Accepted:	04 December 2013

Editor: David del Alamo

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

21 October 2013

Thank you for the submission of your manuscript entitled "Convergent Regulation of Lysosomal Two-Pore Channels by Mg²⁺, NAADP, PI(3,5)P₂ and Protein Kinases" to The EMBO Journal. We have just now received the full set of reports from the referees, which I copy below. As both referees agree on the high interest of your manuscript and their comments are in general positive, I would like to invite you to revise it.

Without going into details that you will find below rather explicitly, both referees consider that your manuscript should be published in The EMBO Journal provided that a number of points -technical concerns for the most part- are addressed in order to improve your message. I would like to draw your attention, however, to points 2 and 3 of Referee #1 and point 2 of referee #2 regarding the physiological relevance of your findings, which would need to be addressed in order to improve your message before final acceptance can be granted.

Please be aware that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will essentially depend on the completeness of your responses included in the next version of the manuscript. Do not hesitate to contact me by e-mail or on the phone in case you have any questions, you need further input or you anticipate any problem during the revision process.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). However, please contact me as soon as possible upon publication of any related work in order to discuss how to proceed. Should you

foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
<http://www.nature.com/emboj/about/process.html>

Thank you very much for the opportunity to consider your work for publication. I look forward to your revision.

REFeree REPORTS

Referee #1

This manuscript provides additional insight into the regulation of a lysosomal ion channel (TPC2). These vacuolar current measurements are not methodologically trivial, and the observation of Mg²⁺/kinase regulation is novel and crucially provides an explanation for the variable ability to record NAADP responses in different cell types. This issue has clear topicality in light of a recent Cell paper concluding TPCs are not regulated by NAADP (Wang et al., referenced). While this study is devoid of molecular data (no data TPC2 is phosphorylated by JNK/p38, or data showing kinase activity is selectively enhanced/inhibited by drug/genetic manipulations), the electrophysiological data (Figs 1-6) is strong. The following experimental issues need to be addressed.

Major.

1. Intact cell calcium imaging (Fig. 7) shows quite a robust NAADP effect, while the electrophysiology data supports a small NAADP-evoked Na⁺ current compared with PI35P2 (Fig 3C, and presumably an even smaller calcium current). However, current measurements uses overexpressed TPC2, but the cell line for imaging expresses predominantly TPC1. Therefore, is the effect of NAADP dependent on the TPC isoforms studied? Are larger currents recorded from TPC1 expressing vacuoles?
2. Correlation between electrophysiological and intact cell data is weak. A dose response curve is needed to demonstrate a similar potency for Mg inhibition in SKBR cells. Ca imaging data needs to be performed with the same pharmacological inhibitors used in the electrophysiological recordings.
3. On balance, this study appears to reaffirm key conclusions of Wang et al. - TPC2 mediated Na⁺ permeability is markedly regulated by PI35P2. In comparison, under optimal and non-physiological conditions (overexpression of TPC2, Mg-free), NAADP evoked a tiny Na⁺ current relative to PI35P2. Evidence for NAADP activation of an endogenous vacuolar current is lacking.
4. It is unclear to me how the single cell imaging data in Figure 7 are generated/compared between manipulations, especially as the authors state that control data is replotted between different graphs. How do the authors know single cells used for Ca imaging are expressing the proposed constructs? This is a concern, as the intact cell data implies overexpression of either kinase potentiates NAADP evoked calcium signals, while knockdown of either blocks responses, even though in either situation only one kinase pathway is presumably affected. While this may reveal a complex phosphoregulation of the channel, it alternatively reveals weakness in processing selected responses from the intact cell dataset.

Minor

1. Do higher doses on NAADP inhibit NAADP-evoked currents (Figure 3c) diagnostic of the NAADP-evoked mechanism?
2. The authors state TPC2 cell surface currents are most readily recordable in cells with intracellular vesicles attached to the cell surface, a circumstantial observation based on Figure 1d alone. Better evidence/images are needed as this may simply be result from higher levels of TPC2 overexpression in these cells, rather than the implied correlation with organelle morphology.

3. Figure 5 legend & Supplementary Figure 3. Please correct drug names.

Referee #2

This is an important study that goes some way to resolve controversies in the two-pore channel (TPC) field. Further, this study extends our understanding of the regulation of TPCs by demonstrating regulation by JNK and p38 kinases as well as Mg ions. They also indirectly show that TPC2 supports calcium fluxes.

Major points:

1. This concerns conventions for ion currents across endomembranes (Bertl et al 1992 Science 258(5084):873-4. Electrical measurements on endomembranes.

Currents flowing into the cytoplasm are termed inward, and conversely those flowing into the lysosomal lumen are termed outward.

Positive charge flowing into the cytoplasm therefore should be termed depolarization.

Clarification of these points in the manuscript would avoid potential confusion.

2. The currents activated by NAADP are small (10%) compared to those activated by PI3P2-could the authors comment please-is this why Wang et al 2012 missed them? What are the physiological significance of these-eg is PI3P2 just a permissive channel factor?

3. The lattice structures in Fig1. Is there any information what organelles form these?

4. p. 3 the permeation to potassium should be discussed-why do TPCs appear to conduct potassium in bilayer studies but not in this (or the Wang et al 2012) study

5. Fig.3b, It is important to show explicitly the individual data points of the NAADP-response curve, this is because the SEM's are so large. Statistical significance should also be stated. Control without NAADP should also be included on the same graph.

Minor points:

1. Abstract 1. 7 insert "a" ... of TPC2 as a PI(35)P2..

2. Cited work: Introduction-perhaps Morgan et al (2011) Molecular mechanisms of endolysosomal Ca²⁺ signalling in health and disease. Biochem J 439, 349-374 should be cited
The work of Biel and colleagues should be cited as evidence that TPC2 forms an NAADP-gated calcium permeant pore Schieder, et al. (2010) Characterization of two-pore channel 2 (TPCN2)-mediated Ca²⁺ currents in isolated lysosomes. J Biol Chem 285, 21219-21222, and Durlu-Kandilci, et al (2010) TPC2 proteins mediate nicotinic acid adenine dinucleotide phosphate (NAADP)- and agonist-evoked contractions of smooth muscle. J Biol Chem 285, 24925-24932 that TPC2 is needed to couple NAADP to calcium release from acidic pools. The work of Pin-Li on TRPML1 and NAADP should be alluded to.

1st Revision - authors' response

22 November 2013

Response to reviewers' comments

Referee #1

This manuscript provides additional insight into the regulation of a lysosomal ion channel (TPC2). These vacuolar current measurements are not methodologically trivial, and the observation of

Mg²⁺/kinase regulation is novel and crucially provides an explanation for the variable ability to record NAADP responses in different cell types. ...the electrophysiological data (Figs 1-6) is strong.

Response: Thank you.

Major.

1. Intact cell calcium imaging (Fig. 7) shows quite a robust NAADP effect, while the electrophysiology data supports a small NAADP-evoked Na⁺ current compared with PI35P2 (Fig 3C, and presumably an even smaller calcium current). However, current measurements uses overexpressed TPC2, but the cell line for imaging expresses predominantly TPC1. Therefore, is the effect of NAADP dependent on the TPC isoforms studied? Are larger currents recorded from TPC1 expressing vacuoles?

Response: The NAADP-mediated Ca²⁺ release is from all organelles expressing NAADP, while the current is measured from fused endolysosomes. The volume of an enlarged endolysosome comprises only a very small fraction of the entire volume of the NAADP-expressing membranes, accounting for the small current but large Ca²⁺ release. As to the relative importance of TPC1 and TPC2, we measured TPC1 current in endolysosomes and in 7 experiments the current is in the same range as that for TPC2. We are not including these results, which are part of a separate study.

2. Correlation between electrophysiological and intact cell data is weak. A dose response curve is needed to demonstrate a similar potency for Mg inhibition in SKBR cells. Ca imaging data needs to be performed with the same pharmacological inhibitors used in the electrophysiological recordings.

Response: Note that the Mg²⁺ inhibition in intact cells is measured by injection of bolus of Mg²⁺ that does not allow as precise control of cytoplasmic Mg²⁺ as in the excised patches. However, to address the comment, in Fig. 7c we now show the effect of injecting two additional Mg²⁺ concentrations showing that at a final concentration of ~ 0.3 mM final concentration of Mg²⁺ inhibited NAADP-mediated Ca²⁺ release by about 60-70%.

As for the effect of kinase inhibitors, note that we used expression of THE SAME P38 and JNK proteins and their dominant negative mutants in both the current and Ca²⁺ release assays. We have now tested the effect of the blockers on NAADP-mediated Ca²⁺ release as requested. We show a potentiating effect, as with the current. The results are given in supplementary Fig. 4.

3. On balance, this study appears to reaffirm key conclusions of Wang et al. - TPC2 mediated Na⁺ permeability is markedly regulated by PI35P2. In comparison, under optimal and non-physiological conditions (overexpression of TPC2, Mg-free), NAADP evoked a tiny Na⁺ current relative to PI35P2. Evidence for NAADP activation of an endogenous vacuolar current is lacking.

Response: Unfortunately the endogenous current is too small to isolate the NAADP activated current. Even with PI(3,5)P₂ which evoked a TPC2 current that is ~10 times larger than that of NAADP, the endogenous current was modest (~150 pA). This makes it impossible to resolve any currents activated by NAADP.

4. It is unclear to me how the single cell imaging data in Figure 7 are generated/compared between manipulations, especially as the authors state that control data is replotted between different graphs. How do the authors know single cells used for Ca imaging are expressing the proposed constructs? This is a concern, as the intact cell data implies overexpression of either kinase potentiates NAADP evoked calcium signals, while knockdown of either blocks responses, even though in either situation only one kinase pathway is presumably affected. While this may reveal a complex phosphoregulation of the channel, it alternatively reveals weakness in processing selected responses from the intact cell dataset.

Response: The control and treated cells are done in the same experiments so that they can be controlled directly. As indicated in the manuscript, TPC2 is tagged with YFP. It is now clarified that when TPC2 is not expressed, cells expressing the kinases and their dominant negatives were identified by co-expression with YFP. No selection was made beyond identifying transfected cells with similar fluorescence intensity.

In the original figure, the controls were replotted since the experiments were performed at the same time. However, these controls were removed and new controls are included that were performed with the new Mg^{2+} experiments.

Minor

1. Do higher doses on NAADP inhibit NAADP-evoked currents (Figure 3c) diagnostic of the NAADP-evoked mechanism?

Response: Desensitization was not observed consistently in the experimental systems used. In the new Fig. 4f we show example of transient responses (consistent with desensitization) observed with 8 endolysosomes and a lack of inactivation observed with 12 lysosomes stimulated with $1 \mu M$ NAADP. These results are described in the manuscript.

2. The authors state TPC2 cell surface currents are most readily recordable in cells with intracellular vesicles attached to the cell surface, a circumstantial observation based on Figure 1d alone. Better evidence/images are needed as this may simply be result from higher levels of TPC2 overexpression in these cells, rather than the implied correlation with organelle morphology.

Response: We agree that higher TPC2 expression can account for the higher current and the statement was removed.

3. Figure 5 legend & Supplementary Figure 3. Please correct drug names.

Response: Thank you. This was corrected.

Referee #2

This is an important study that goes some way to resolve controversies in the two-pore channel (TPC) field. Further, this study extends our understanding of the regulation of TPCs by demonstrating regulation by JNK and p38 kinases as well as Mg ions. They also indirectly show that TPC2 supports calcium fluxes.

Response: Thank you.

Major points:

1. This concerns conventions for ion currents across endomembranes (Bertl et al 1992 Science 258(5084):873-4). Electrical measurements on endomembranes. Currents flowing into the cytoplasm are termed inward, and conversely those flowing into the lysosomal lumen are termed outward. Positive charge flowing into the cytoplasm therefore should be termed depolarization. Clarification of these points in the manuscript would avoid potential confusion.

Response: Thank you. We accepted the argument and change all outward to inward and vice versa. However, please note that this does not change the effect of opening the channel on the membrane potential, which is determined only by the Na^+ gradients across the endolysosomal membrane.

2. The currents activated by NAADP are small (10%) compared to those activated by $PI(3,5)P_2$ -could the authors comment please-is this why Wang et al 2012 missed them? What are the physiological significance of these-eg is $PI(3,5)P_2$ just a permissive channel factor?

Response: It is difficult to know why Wang et al missed the NAADP response. It is possible that both, the size of the current and including Mg^{2+} in all their solutions contributed to missing the response. However, in the manuscript we describe our data with some reference to the Wang papers, but we think it is for Wang et al to re-examine this issue.

We now discuss the potential physiological significance of the difference between the size of the current activated by $PI(3,5)P_2$ and NAADP. As you indicate, we believe that $PI(3,5)P_2$ is a permissive factor while NAADP is the physiological activator of the channel. The strongest

evidence for this is the results in intact cells, where manipulation of PI(3,5)P₂ changes the size of the NAADP response rather than eliminates it. Thus, likely changes in endolysosomal PI(3,5)P₂ regulate the size of the NAADP response but do not serve as the trigger of the channel.

3. The lattice structures in Fig1. Is there any information what organelles form these?

Response: We do not have any information on this at this time.

4. p. 3 the permeation to potassium should be discussed-why do TPCs appear to conduct potassium in bilayer studies but not in this (or the Wang et al 2012) study

Response: We can only speculate that the lipid composition of the artificial bilayers may change the channel selectivity. This is now mentioned in the manuscript.

5. Fig.3b, It is important to show explicitly the individual data points of the NAADP-response curve, this is because the SEM's are so large. Statistical significance should also be stated. Control without NAADP should also be included on the same graph.

Response: We now show the individual current measurements in the form of a scatter plot. However, we still include the columns in which the currents are expressed as a % of PI(3,5)P₂ for normalization. The normalization is required to show the more accurate concentration dependence of channel activation by NAADP. The results were also re-calculated. The original calculation was done during early stage of the studies before we realized that only about 50% of patches respond to NAADP and thus all experiments, including the experiments in which current was not observed were included in the original calculation. The current calculation includes only patches showing an NAADP-activated current. We do not include the current before NAADP since the leak current is variable and was subtracted from all records and thus we only show the NAADP-activated current. Statistical significance is not included since unfortunately there is not statistically significant difference between the currents. However, we now indicate in the text that the statistical difference between 3nM/10nM, 3nM/1 μM, and 10nM/1 μM was between 0.09-0.13.

Minor points:

1. Abstract l. 7 insert "a" ... of TPC2 as a PI(35)P₂..

Response: Thank you. This was corrected in the revised shorter abstract.

2. Cited work: Introduction-perhaps Morgan et al (2011) Molecular mechanisms of endolysosomal Ca²⁺ signalling in health and disease. Biochem J 439, 349-374 should be cited
The work of Biel and colleagues should be cited as evidence that TPC2 forms an NAADP-gated calcium permeant pore Schieder, et al. (2010) Characterization of two-pore channel 2 (TPCN2)-mediated Ca²⁺ currents in isolated lysosomes. J Biol Chem 285, 21219-21222, and Durlu-Kandilci, et al (2010) TPC2 proteins mediate nicotinic acid adenine dinucleotide phosphate (NAADP)- and agonist-evoked contractions of smooth muscle. J Biol Chem 285, 24925-24932 that TPC2 is needed to couple NAADP to calcium release from acidic pools. The work of Pin-Li on TRPML1 and NAADP should be alluded to.

Response: Thank you. All references are now cited.

2nd Editorial Decision

02 December 2013

Thank you for the submission of your revised manuscript to The EMBO Journal. Your study was sent back to former referee #2, who now believes that all major concerns have been properly addressed and your manuscript is almost ready for publication. That being said, a few minor issues that still require your attention, mostly related to the presentation of your data, will have to be addressed. Once these minor problems have been solved, I will be glad to accept your manuscript for publication in The EMBO Journal.

As you will see below, the referee correctly points out to the fact that Figure 4f has not been provided in the revised version of the manuscript. In addition, browsing through the manuscript myself, I have noticed that Supplementary Figure S3 is mislabeled as S2 in the Supplementary figure legends.

More generally, I have noticed that the micrographs in Figure 1 lack scale bars, which we require for clarity. Furthermore, the statistical analysis of the results throughout the study, including supplementary information, is insufficiently described. As a guide, statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply and will include a definition of the error bars used and the number of independent experiments performed. The statistical significance analysis tool used, if any, must be also clearly stated.

Every paper now includes a 'Synopsis' to further enhance their discoverability. Synopses are displayed on the html version of the article and they are freely accessible to all readers. The synopsis includes an image, normally cropped by us from one of the final figures of the manuscript, as well as 2-5 one-sentence bullet points that summarize the article and should be complementary to the abstract - i.e. not repeat the same text. Could I ask you to provide the bullet points as a separate word file as part of your final manuscript?

Please, do not hesitate to contact me in case you have any questions.

Thank you very much for your patience. I am looking forward to seeing the final version of your manuscript.

REFeree REPORT

Referee #2:

The revised version of the manuscript provides new experimental data to address the majority of comments raised in the original review. I think this is a highly topical study that is of importance to the calcium field given recent high profile studies (Cang et al/ Wang et al published in Cell) suggesting TPCs are not the target for NAADP action. This study should therefore be of wide interest, and trigger further debate as to the role of TPCs in cellular signaling.

Minor point - in response to Minor Point #1 of my review, the authors talk about a new Figure 4F. This figure is not present in the submitted revised version and needs to be included. Obviously, I do not need to see this revision to facilitate timely publication.

2nd Revision - authors' response

03 December 2013

As you will see below, the referee correctly points out to the fact that Figure 4f has not been provided in the revised version of the manuscript.

Response: The mention of Fig. 4f is an error. Originally, the results in the new supplementary Fig. 3 were included in the manuscript as Fig. 4f. However, this broke the flow of the text and thus they were moved to the supplement. In error, the text in the response was not changed.

In addition, browsing through the manuscript myself, I have noticed that Supplementary Figure S3 is mislabeled as S2 in the Supplementary figure legends.

Response: Thank you. This was corrected.

More generally, I have noticed that the micrographs in Figure 1 lack scale bars, which we require for clarity.

Response: Thank you. Scale was added to all images.

Furthermore, the statistical analysis of the results throughout the study, including supplementary information, is insufficiently described. As a guide, statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply and will include a definition of the error bars used and the number of independent experiments performed. The statistical significance analysis tool used, if any, must be also clearly stated.

Response: Thank you. A paragraph was added to the result section that reads: *Statistics:* All experiments were repeated at least three times and the results are given as means \pm s.e.m. Differences between the groups were analyzed for statistical significance by non-paired student's t-test. $P < 0.05$ or better was considered statistically significant.